CEFAZOLIN, A NEW SEMISYNTHETIC CEPHALOSPORIN ANTIBIOTIC. VI

EXCRETION AND METABOLISM OF CEFAZOLIN-^{14}C IN RATS AFTER INTRAMUSCULAR ADMINISTRATION*

JUN KOZATANI, MASAO OKUI, TADAO MATSUBARA and MINORU NISHIDA

Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan

(Received for publication September 22, 1971)

Cefazolin is a new semisynthetic cephalosporin antibiotic. The urinary and biliary excretion and tissue levels of cefazolin were determined in rats by a simultaneous assaying of the biological and radiological activity following intramuscular injection of ^{14}C-labeled cefazolin sodium to investigate the metabolism *in vivo*. The results of both determinations coincided in many aspects. Paper chromatography and subsequent autoradiography and bioautography revealed that the great majority of urinary and biliary radioactivities were derived from unchanged cefazolin. Thin-layer chromatography and inverse isotope dilution analysis indicated the same fact. These data proved that there is little biotransformation of parenterally administered cefazolin in the animal body.

The preceding paper[1] concerning the metabolic fate of cefazolin-^{14}C (CEZ-^{14}C) demonstrated the distribution of radioactivity in rats and mice after parenteral administration. The present investigation aims to elucidate the problem of questionable *in vivo* metabolism of this antibiotic, by comparing simultaneously obtained data on antibiotic activity and radioactivity of the same sample (urine, bile and tissue) after an intramuscular administration of sodium salt of CEZ-^{14}C. Chromatographic analysis and the inverse isotope dilution method also contributed to the investigation.

Materials and Methods

^{14}C-Labeled cefazolin: The labeled antibiotics used were sodium 7-[1-(1H)-tetrazolylacetamido-2-^{14}C]-3-[2-(5-methyl-1, 3, 4-thiadiazolyl)-thiomethyl-Δ^3-cephem-4-carboxylate (CEZ-Na-7-^{14}C, with a specific radioactivity of 4.70 μCi/mg and radiochemical purity of 93.0 %) and sodium 7-[1-(1H)-tetrazolylacetamido]-3-[2-(5-methyl-1,3,4-thiadiazolyl)-thiomethyl-2-^{14}C]-Δ^3-cephem-4-carboxylate (CEZ-Na-3-^{14}C, with a specific radioactivity of 3.80 μCi/mg and radiochemical purity of 90.2 %). These were the same compounds as used in the experiment of the preceding paper[1].

Animal studies: Sprague-Dawley-JCL male rats, weighing 240~310 g, were injected about 20 mg/kg of any of the CEZ-Na-^{14}C dissolved in physiological saline, which had been diluted with the standard preparation of the unlabeled antibiotic so that each animal might receive 1.5~3 μCi of radioactivity. Four rats were restrained in a prone

* Studies on the metabolism of cephalosporins. IV.

position on surgical boards, and the urine samples were collected into conical glass tubes of 10-ml capacity, graduated to 0.1 ml, through a small glass funnel affixed to the orifice of the urethra. Feces on the surgical boards were also collected. Bile was collected by the procedures described before[2]. The carcasses were examined for radioactivity by the method described in the preceding paper[1].

For the simultaneous investigation of antibiotic activity and radioactivity in organs and tissue, rats were divided into groups of 3 animals each and, after dosing, sacrificed at different times by exsanguination at the femoral artery and vein. Organs were removed, rinsed with physiological saline, blotted dry, and pooled organs were each homogenized using a Virtis-45 homogenizer under ice-cooling for two minutes. Homogenates used were made from the following organs each contained in specified volumes of 99% ethanol: brain, in 2 volumes; heart, spleen, and liver, in 3 volumes; and kidneys, in 4 volumes. Aliquots of these homogenates were each centrifuged at 8,000 r.p.m. for 15 minutes under cooling at 5°C, and the supernatant fluids were divided and subjected to bioassay and radioassay respectively. Serum was also subjected to both analyses.

Measurement of radioactivity: The procedures described in the preceding paper[1] were employed for urine, bile, serum, whole blood, feces and carcasses. A 100-μl aliquot of each supernatant fluid obtained above was added to one ml of Soluene-100 (Packard Instrument Co.) and to 15 ml of toluene scintillator. After development, the paper strips were dissected at 1-cm intervals from the starting point, and transferred into counting vials, to which were subsequently added 100 μl of 1N NaOH, 3 ml of methanol, and finally 10 ml of the toluene scintillator. Areas of radioactivity on the developed TLC plates, located by autoradiography, were scraped into separate counting vials. Subsequently the aforementioned volumes of NaOH, methanol, and the scintillator were similarly added.

The radioactivity was measured by a Tri-Carb 3375 and a 3380 liquid scintillation spectrometer (Packard Instrument Co.), and the external standard channels ratio method was employed for quenching correction.

Measurement of antibiotic activity: The antibiotic activity in the samples was determined by the disk method using *Bacillus subtilis* ATCC 6633 as the test organism. The serum and bile specimens were bioassayed by referring to standard dilutions consisting of 9 volumes of the control serum or bile and one volume of the CEZ solution.

Chromatographic procedure:

1) Paper chromatography: Paper chromatography was carried out with Toyo No. 51 filter paper (2×40 cm). Undiluted urine and bile samples were applied to the paper in 2~10 μl, then developed in the solvent system of *n*-butanol - acetic acid - water (4 : 1 : 2), using an ascending technique for 16 hours at room temperature.

2) Thin-layer chromatography: Urine and bile were applied to silica gel plates F_{254} (0.25 mm thickness, 20×20 cm with fluorescein indicator, Merck Co.), developed in two different solvent systems (*n*-BuOH - HOAc - H_2O, 4 : 1 : 2 and EtOAc - HOAc - H_2O, 4 : 2 : 1) for a length of about 10 cm.

3) Autoradiography: The developed paper strips and silica gel plates were exposed to X-ray films for one week by the contact method. However, some paper strips were exposed for an additional one week. The films were developed in a Konidol-X developer for 5 minutes and fixed in Konifix for 15 minutes.

4) Bioautography: After exposure for one week, the paper strips were placed on agar plates (20×30 cm) seeded with *B. subtilis* ATCC 6633 for 30 minutes at 5°C. After removing the strips, the plates were incubated overnight at 37°C. Areas of antibiotic activity were located by inhibition of growth.

Inverse isotope dilution method: An aliquot (0.5~1.0 ml) of urine or bile specimen was added to about 500 mg of accurately weighed CEZ-Na. To this mixture were added water to dissolve, and then a small amount of acetone until the solution was slightly turbid. Subsequently this solution was cooled, and the inner wall of the vessel was

rubbed with a spatula to achieve crystallization. The crystals were collected by filtration, washed with a small volume of acetone, and dried over phosphorous pentoxide *in vacuo* for 3 hours. About 5~10 mg of the crystal was accurately weighed, dissolved by adding 100 μl of water, 3 ml of methanol, and 10 ml of toluene scintillator, and counted for radioactivity. Recrystallization was repeated several times until constant specific activity was attained. The relative amount of unmetabolized CEZ present in the original samples was calculated by the formula:

$$X=\frac{Sm}{b}\times 100 \qquad (I)$$

where, S: specific activity of the isolate
m: weight of unlabeled CEZ-Na added
b: total radioactivity of original sample
X: % of unmetabolized CEZ content

Since the crystalline unlabeled CEZ-Na obtained from the same procedure revealed to contain about 2.7 % water when analyzed by KARL-FISCHER's method, formula (I) was corrected on the basis of the water content, and additionally of radiochemical purity of authentic CEZ-Na-7-^{14}C (91.3 %) and CEZ-Na-3-^{14}C (92.8 %) to determine the unmetabolized CEZ content.

Serum protein binding: Non-fasted male rats were divided into groups of 3 animals each, and CEZ-Na-7-^{14}C (20 mg/kg) was injected intramuscularly. Animals were sacrificed at different times by exsanguination at the femoral artery and vein. A 2-ml portion of serum was transferred into Visking tubing (8/32) about 15 cm long, which had been soaked in boiling distilled water for 1 hour, and subjected to ultrafiltration by centrifuging at 3,500 r.p.m. (about 1,500 g) for 60 minutes at 5°C. It produced about 0.4 ml of ultrafiltrate. Biological and radiological activities in the serum and in the ultrafiltrate were determined, and used to calculate the amounts of CEZ bound to serum protein. To correct any possible adsorption of the antibiotic to the membrane or other variable factors, a suitable phosphate buffer solution (pH 7.4, M/15, Na and K) of CEZ-Na-7-^{14}C was treated similarly. The relative amount of protein bound CEZ in the serum was calculated by the formula:

$$B=\frac{Ts-Fs}{Ts}\times 100-\frac{Tc-Fc}{Tc}\times 100$$

where, Ts: total drug in serum (mcg/ml or dpm/ml)
Fs: free drug in ultrafiltrate of serum (mcg/ml or dpm/ml)
Tc: total drug in control buffer solution (mcg/ml or dpm/ml)
Fc: free drug in ultrafiltrate of control buffer solution (mcg/ml or dpm/ml)
B: percent protein binding

Results and Discussion

Urinary and Fecal Excretion

Table 1 shows the recovery of total urinary and fecal radioactivity and antibiotic activity of rats after a single intramuscular injection of CEZ-Na-7-^{14}C (20 mg/kg). These results show that the numerals from both assay methods are consistent and that the appearance of this antibiotic in the urine is very fast as is evident by the numerals of 70 % of the administered dose in 3 hours, 80 % in 6 hours, and 82 % or more in 24 hours. The urinary recovery during 24 hours accounted for 82 % in the bioassay and 84 % in the radioassay, indicating no significant difference between the two assays (t-test, $p<0.05$). Fecal recovery of radioactivity in 24 hours accounted for about 10 %. The carcasses showed radioactivity equal to 3.5 % that of the administered radioactivity. Those data are indicative of little metabolism of this antibiotic in the rat body.

Table 1. Urinary and fecal excretion of CEZ-Na-7-^{14}C in rats injected intramuscularly with 20 mg/kg (mean±standard error; n=4)

Assay		0~3 hour		3~6 hour		6~24 hour		Cumulative excretion % (0~24 hour)
		Conc. (mcg/ml)	% of dose	Conc. (mcg/ml)	% of dose	Conc. (mcg/ml)	% of dose	
Urinary	Bio-	2,461 ±580	73.07 ±3.41	335.8 ±77.9	7.15 ±1.19	13.4 ±3.2	1.44 ±0.33	81.78±3.31
	Radio-	2,352 ±570	69.91 ±4.31	432.2 ±68.8	9.39 ±1.62	43.2 ±6.9	4.83* ±0.74	84.13±3.09
	Bio-/Radio-ratio	1.05		0.76		0.30		0.97
Fecal (Radio activity %)		0.04±0.01		1.01±0.95		8.69±1.97		9.75±2.22
Total		69.95±4.31		10.40±1.76		13.52±2.29		93.87±1.36**

* Bioassay<Radioassay (t-test, $p<0.05$)
** 3.46±0.80% of administered radioactivity remained in carcasses of rats sacrificed at the termination of the experiment.

Table 2. Biliary and urinary excretion of CEZ-Na-7-^{14}C in rats injected intramuscularly with 20 mg/kg (mean±standard error; n=4)

Assay		0~3 hour		3~6 hour		6~24 hour		Comulative % (0~24 hour)
		Conc. (mcg/ml)	% of dose	Conc. (mcg/ml)	% of dose	Conc. (mcg/ml)	% of dose	
Biliary	Bio-	332.5 ±36.4	17.96 ±2.36	46.3 ±15.2	1.80 ±0.54	2.4* ±1.0	0.35 ±0.13	20.11±2.38
	Radio-	351.7 ±51.5	18.73 ±2.31	50.1 ±17.4	1.92 ±0.61	2.3±0.9	0.33 ±0.10	20.98±2.61
	Bio-/Radio-ratio	0.96		0.94		1.06		0.96
Urinary	Bio-	2,640 ±919	30.14 ±10.75	1,356 ±323	28.61 ±12.76	51.4 ±12.2	7.55 ±2.17	66.30±2.74
	Radio-	2,383 ±833	27.11 ±9.60	1,257 ±316	26.96 ±12.60	38.0 ±9.0	6.10 ±1.34	60.17±3.95
	Bio-/Radio-ratio	1.11		1.06		1.24		1.10
Total	Bio-		48.10 ±12.88		30.41 ±12.54		7.90 ±2.28	86.40±1.56
	Radio-		45.84 ±11.69		28.88 ±12.34		6.42 ±1.43	81.15±1.68**
	Bio-/Radio-ratio	1.05		1.05		1.23		1.06

* One rat showed no assayable concentration (<0.7 mcg/ml), so estimated as 0.7 mcg/ml.
** 2.43±1.46% of administered radioactivity remained in carcasses of rats sacrificed at the termination of the experiment.

Biliary Excretion

Table 2 shows the biliary and urinary excretion of antibiotic activity as well as radioactivity over a 24-hour period after a single intramuscular injection of CEZ-Na-7-^{14}C into four canulated rats. About 18% of the administered dose, in radioassay as well as in bioassay, was recovered in the 3-hour bile. The cumulative excretion after 24 hours accounted for 20% in the bioassay and 21% in the radioassay, indicating no significant difference. The carcasses of rats sacrificed at 24 hours after administration retained 2.4% of administered radioactivity. This comparison infers that CEZ was excreted into the bile largely in the unchanged form.

Tissue Levels

Male rats were divided into groups of 3 animals each, and CEZ-Na-7-^{14}C (20 mg/kg) was given intramuscularly for subsequent determination of the tissue levels. Table 3 shows the tissue levels of antibiotic activity as well as radioactivity during 2 hours after administration. Throughout the 2-hour assay, CEZ levels were notably high in the serum and kidneys, moderate in the liver, lungs, and heart, and low in the spleen and brain. These levels peaked at 15 minutes and then declined, keeping a close relationship between the matched values. These data suggest that no extensive biotransformation of CEZ occurred in the rat body. Plotting these data on semi-logarithmic paper yields curves that fit the kinetics of first-order elimination in most organs. The slopes of the best straight lines were obtained by computer regression analysis and used to estimate the half-lives of each assay method in the organs (Table 3).

There was a fairly good agreement between the present data and the data in the preceding paper[1] except that most tissue levels in the present data are slightly lower, as was elucidated by the fact that the precipitate of each organ homogenate retained a little radioactivity. The serum and supernatant fluids of homogenates of liver and kidneys at 30 minutes were analyzed for the content of unchanged CEZ with the inverse isotope dilution method. The CEZ content was 88.0 % in the serum, 93.2 %

Table 3. Tissue levels of CEZ-Na-7-^{14}C (mcg/g) in rats injected intramuscularly with 20 mg/kg

Organs from 3 rats were each pooled and homogenized in ethanol. Supernatant of the homogenate was subjected to the assay (see text).

Time	Assay method	Brain	Heart	Lungs	Spleen	Liver	Kidneys	Whole blood**	Serum**
15 min.	Bio-	n. d.	12.7	21.0	5.1	22.5	75.5	—	—
	Radio-	1.1	14.4	19.5	5.1	22.4	68.6	50.96±0.99	77.60±2.21
30 min.	Bio-	n. d.	9.6	10.1	4.0	22.2	52.7	—	59.8 ±3.9
	Radio-	1.0	9.9	13.4	5.1	24.6	57.3	—	58.58±3.41
1 hour	Bio-	n. d.	5.4	8.7	2.9	5.8	20.9	—	31.6 ±2.8
	Radio-	0.5	6.3	9.3	2.8	6.8	26.2	18.14±1.69	31.43±2.57
2 hour	Bio-	n. d.	n. d.	4.4	n. d.	2.9	6.5	—	8.8 ±2.6
	Radio-	0.2	1.8	5.1	1.1	1.6	8.6	6.44±1.98	10.30±3.36
Half-life (min.)	Bio-	—	36.4	***	***	33.1	29.6	—	32.6
	Radio-	41.1	35.6	57.2	44.8	25.6	34.2	***	35.9

* Not detected (<0.7 mcg/g).
** mcg/ml, mean±standard error.
*** Did not fit the kinetics of first-order (t-test, $p<0.05$).
— : Not determined.

Table 4. Serum protein binding* (%) of CEZ-Na-7-^{14}C in rats injected intramuscularly with 20 mg/kg

Assay	5 minutes	15 minutes	30 minutes	60 minutes	120 minutes
Bio-	73.94±1.27**	92.49±0.85	88.90±0.98	88.11±0.62	82.17±3.04
Radio-	81.23±0.66	82.79±1.17	89.34±1.11	88.09±0.47	73.61±8.16

* Determined by ultrafiltration.
** Mean±standard error (n=3).

in the liver, and 97.3% in the kidneys respectively.

Serum Protein Binding

The percentages of CEZ bound to the rat serum, determined by both assay methods, are shown in Table 4. CEZ was highly bound throughout the investigation. *In vitro* incubation of CEZ solution with pooled rat serum at 37°C for one hour showed 72% protein binding.

Metabolism in Urine and Bile

The urinary and biliary metabolic patterns of CEZ were examined by the chromatographic and inverse isotope dilution methods in the three canulated rats, after injecting 10 mg/kg of CEZ-Na-7-^{14}C or CEZ-Na-3-^{14}C intramuscularly (11~15 μCi/rat).

The autoradiographic patterns of urinary and biliary radioactivity is shown in Figs. 1 and 2. The blackened areas represent localization of radioactivity. These autoradiograms show that CEZ is excreted into urine and bile mostly unchanged, with traces of unidentified metabolites. Each paper strip was divided at 1-cm intervals, and the radioactivity was measured with a liquid scintillation spectrometer to determine the relative amount in each sample. The radiochemical purity of authentic CEZ-Na-7-^{14}C and CEZ-Na-3-^{14}C was 89.7% and 91.0%, respectively. After administration of CEZ-Na-7-^{14}C, unidentified metabolites in the 0~4 hour bile were more polar than CEZ, and accounted for 3.5%

Fig. 1. Autoradiogram of rat urine and bile after intramuscular injection of CEZ-Na-7-^{14}C (10 mg/kg) [Rat No. 1].

Paper chromatography: Toyo No. 51 filter paper (2×40 cm). *n*-BuOH-HOAc-H_2O (4:1:2). Ascending method (for 16 hours) exposed to X-ray film for 14 days.

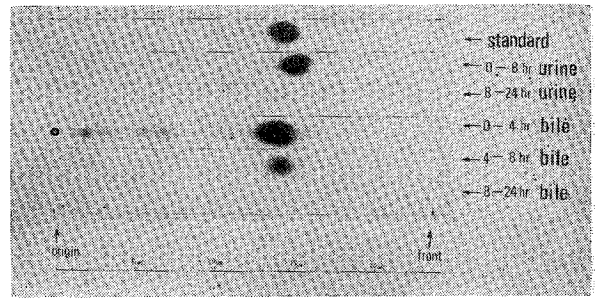


Fig. 2. Autoradiogram of rat urine and bile after intramuscular injection of CEZ-Na-3-^{14}C (10 mg/kg) [Rat No. 2].

Paper chromatography: Toyo No. 51 filter paper (2×40 cm). *n*-BuOH-HOAc-H_2O (4:1:2). Ascending method (for 16 hours) exposed to X-ray film for 14 days.

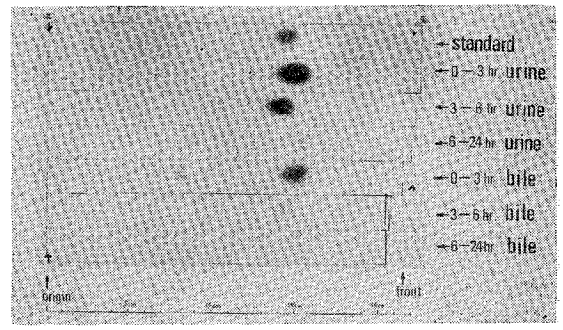
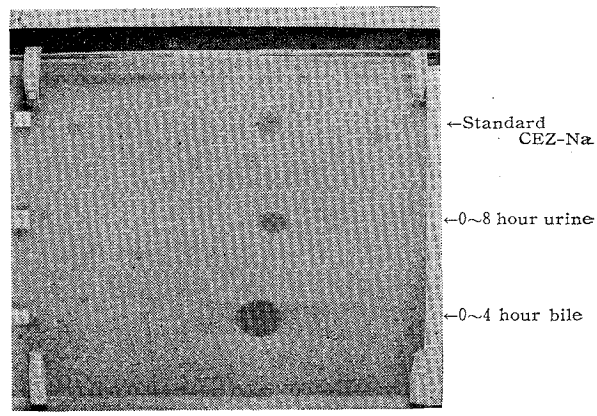


Fig. 3. Bioautograms of rat urine and bile after intramuscular injection of CEZ-Na-7-^{14}C (10 mg/kg).

Paper chromatography: Toyo No. 51 filter paper (2×40 cm). *n*-BuOH-HOAc-H_2O (4:1:2). Ascending method (for 16 hours).

Test organism: *B. subtilis* ATCC 6633



(Rf 0.09), 2.8% (Rf 0.14), 2.2% (Rf 0.24), 3.6% (Rf 0.30) and 3.8% (origin), respectively. After administration of CEZ-Na-3-^{14}C, other radioactive metabolites in the 0~3-hour bile represented 8.6% (Rf 0.38) and 4.9% (Rf 0.96) of the biliary radioactivity, and 0.6% (Rf 0.39) and 1.2% (Rf 0.73) of the urinary radioactivity.

The bioautograms are shown in Figs. 3 and 4. Only one biologically active spot, the Rf value of which was consistent with that of the authentic CEZ, was found in the urine and bile. The radioactive metabolites in the urine and bile were considered to be biologically inactive.

Fig. 4. Bioautograms of rat urine and bile after intramuscular injection of CEZ-Na-3-^{14}C (10 mg/kg).

Paper chromatography: Toyo No. 51 filter paper (2×40 cm). *n*-BuOH-HOAc-H_2O (4:1:2). Ascending method (for 16 hours).
Test organism: *B. subtilis* ATCC 6633

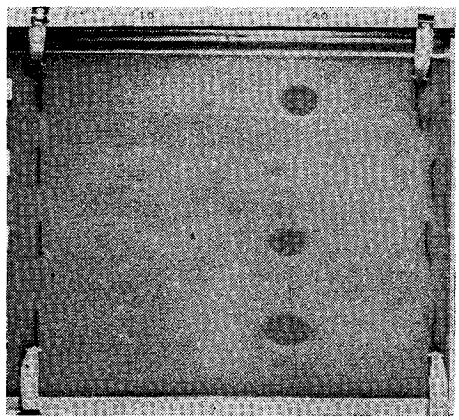


Table 5. Urinary and biliary excretion of unchanged CEZ analyzed by three methods (% of the administered dose)

Time (hours)		CEZ-Na-7-^{14}C (Rat No. 1)			CEZ-Na-3-^{14}C (Rat No. 3)		
		Total ^{14}C excretion %	Unchanged CEZ %		Total ^{14}C excretion %	Unchanged CEZ %	
			PPC	IDA		TLC	IDA
Urine	0~4	71.64	71.6	68.5	42.93	42.9	34.8
	4~8				14.17	14.0	13.1
	8~24	1.53	1.2	1.3	3.71	—	—
	Total	73.17	72.8	69.8	60.81	56.9	47.9
Bile	0~4	27.87	21.0	27.9	25.34	21.2	23.1
	4~8	0.29	0.2	0.3	1.75	1.1	1.5
	8~24	1.53	—	—	0.22	—	0.2
	Total	29.69	21.2	28.2	27.31	22.3	24.8
Total recovery		102.86	94.0	98.0	88.12	79.2	72.7

—: Unchanged CEZ could not be determined.
PPC: Paper chromatography. TLC: Thin-layer chromatography.
IDA: Inverse isotope dilution analysis.

Furthermore, the amount and nature of the radioactive metabolites in the urine and bile were determined by thin-layer chromatography and subsequent autoradiography and counting by a liquid scintillation spectrometer. The results are summarized in Table 5. The amount of unchanged CEZ-Na-3-^{14}C excreted in the urine and bile was slightly lower than in the case of CEZ-Na-7-^{14}C. This was attributable to the lower recovery of total radioactivity than that of the latter, and not to the proportion of unchanged drug in the samples.

The results of these studies indicate that, when CEZ is administered intramuscularly into rats, it does not undergo extensive metabolism, and is rapidly excreted into urine and bile mostly in the unchanged form.

Acknowledgement

The authors wish to thank Dr. I. NAKAYAMA of Nihon University for many helpful discussions and suggestions during this work, and also Dr. H. NAKANO, the director of this research laboratories, for guidance and encouragement.

References

1) KOZATANI, J.; M. OKUI, K. NODA, T. OGINO & H. NOGUCHI: Cefazolin, a new semisynthetic cephalosporin antibiotic. V. Distribution of cefazolin-^{14}C in mice and rats after parenteral administration. Chem. Pharm. Bull. (Tokyo) submitted for publication
2) OKUI, M.; K. HATTORI & M. NISHIDA: Studies on the metabolism of cephalosporins. II. Biliary excretion and metabolism of ^{14}C-cephachlomezine and its parent compound. J. Antibiotics 24: 667~674, 1971
3) NISHIDA, M.; T. MATSUBARA, T. MURAKAWA, Y. MINE, Y. YOKOTA, S. GOTO & S. KUWAHARA: Cefazolin, a new semisynthetic cephalosporin antibiotic. II. *In vitro* and *in vivo* antimicrobial activity. J. Antibiotics 23: 137~148, 1970
4) NISHIDA, M.; T. MATSUBARA, Y. MINE, T. MURAKAWA, Y. YOKOTA, S. GOTO & S. KUWAHARA: Cefazolin, a new semisynthetic cephalosporin antibiotic. III. Absorption, excretion and tissue distribution in parenteral administration. J. Antibiotics 23: 184~194, 1970